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A Plasmid in *Legionella pneumophila*

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Sixteen strains from the six serogroups of *Legionella pneumophila* were examined for the presence of extrachromosomal genetic elements by a modified cleared lysate procedure, dye-buoyant centrifugation, and agarose gel electrophoresis. Two strains, Atlanta-1 and Atlanta-2 from serogroup II, each contained a plasmid of cryptic function with a molecular weight of ca. 30 megadaltons.

Legionella pneumophila (2) is the etiological agent of legionellosis, which has two markedly different clinical presentations: Legionnaires disease, a pneumonic form with a significant case fatality rate, and Pontiac fever, a nonpneumonic form which is not fatal. During the July 1976 outbreak of legionellosis at the Bellevue-Stratford Hotel in Philadelphia, 221 people became ill, of whom 34 died from pneumonia or its complications (6). More than 600 cases of legionellosis have now been reported from more than 40 states of the United States, Australia, Canada, Denmark, England, Greece, Israel, Italy, The Netherlands, Norway, Scotland, Spain, Sweden, West Germany, and Yugoslavia. It has been estimated that there are approximately 26,000 undiagnosed cases of legionellosis in the United States per year (13).

The Legionnaires disease bacterium, *L. pneumophila*, is a fastidious, aerobic, gram-negative non-acid-fast, nonmotile, rod-shaped bacterium ca. 0.3 to 0.5 μm wide and 2 to 20 μm in length, which can not be cultivated on most bacteriological media (22). It can be grown on Mueller-Hinton agar supplemented with 1% hemoglobin and 1% IsoVitaleX (BBL Microbiology Systems, Cockeysville, Md.) (7), on charcoal-yeast extract (CYE) agar (12), or in yeast extract broth (27), each of which is supplemented with cysteine and iron. *L. pneumophila* has been isolated from soil samples and from air-conditioning cooling water, but its precise ecological niche and nutritional supply have not been determined.

Although controlled human chemotherapeutic trials on legionellosis have not been carried out, in vitro studies have shown that the causative organism is susceptible to a number of antibiotics (28, 30). Many of these antibiotics can be rendered ineffective by naturally occurring drug resistance plasmids, which are found in strains of *Escherichia coli* (25). Transfer of these resistance plasmids to *L. pneumophila* could produce problems in antibiotic therapy. It is known that *L. pneumophila* produces a β-lactamase, which is more active on cephalosporin than on penicillin (29, 31). Since β-lactamase-specifying plasmids have been identified from a broad taxonomic range of bacteria (11, 18) and β-lactamase production in *L. pneumophila* could be due to the presence of antibiotic resistance plasmids, we have examined the deoxyribonucleic acid (DNA) from a number of *L. pneumophila* strains for the presence of plasmids.

MATERIALS AND METHODS

Bacterial strains. *E. coli* V517, kindly supplied by the Plasmid Reference Center, Stanford University, Stanford, Calif., contains eight plasmid species ranging in size from 1.36 to 35.8 megadaltons (21).

There are six recognized serogroups of *L. pneumophila* (10, 23). Strains are designated by the city in which they were isolated and the number of the isolate from that city. The following 16 representative strains from the six serogroups were examined: serogroup I, Philadelphia-1 through -4, Flint-1, Knoxville-1, Washington-1, Vermont-1, and Pontiac-1; serogroup II, Atlanta-1 and -2 and Togas-1; serogroup III, Bloomington-2; serogroup IV, Los Angeles-1; serogroup V, Dallas-4; and serogroup VI, Chicago-2. These strains had been isolated from patients with legionellosis or from environmental samples (3), inoculated into guinea pigs, and passed into the yolk sacs of embryonated eggs. Atlanta-1 and -2 were initially isolated from human lung specimens in 1978 and were preserved on CYE slants. Isolates were obtained from the Center for Disease Control, Atlanta, Ga. All manipulations with these organisms were conducted in a maximum containment facility.

Media and culture conditions. Stock cultures of *L. pneumophila* were maintained in homogenated yolk sacs of embryonated hen eggs stored at −70°C. Cultures were routinely grown at 37°C on CYE agar (12) and in yeast extract broth (27). *L. pneumophila* yeast extract broth cultures of 400 ml in 1-liter Erlenmeyer flasks were incubated at 37°C on a New Brunswick reciprocating incubator shaker (100 strokes per min). The cultures were monitored for contamination by plating on blood agar and by microscopic examination. Minimal medium M9 (1), supplemented with 0.2% glucose and 1% Casamino Acids, was used to grow all *E. coli* cultures.

Preparation of cleared lysates. Various methods of preparing lysates were employed on strains of *L. pneumophila*; the following optimized procedure was established as a modification of the method of Clewell and Helinski (8). Cells were harvested by scraping 10 CYE agar plates, which exhibited confluent growth, using a bent glass rod and 3 ml per plate of saline containing 0.1% tryptose, pH 7.0. Each strain was also harvested from 400 ml of yeast extract broth culture at ca. 2×10^9 cells/ml by centrifugation at 10,000 × *g* for 10 min at 4°C. The pellet was resuspended in 25 ml of 25% sucrose solution made up in 0.05 M tris(hydroxymethyl)aminomethane (Tris), pH 8.0, at room temperature. Spheroplasts were generated by the addition of 3.0 ml of freshly prepared lysozyme solution (10 mg/ml in 0.25 M Tris, pH 8.0). The suspension was mixed and placed on ice for 5 min. A 3.0-ml portion of 0.25 M ethylenediaminetetraacetate (EDTA), pH 8.0, was then added, and the mixture was chilled in an ice bath for an additional 5 min with occasional swirling. Lysis was brought about by adding 30 ml of 2.0% Triton X-100 made up in 0.025 M EDTA and 0.05 M Tris, pH 8.0. The final mixture was incubated at 37°C for 15 min and gently swirled until clearing occurred. The viscous lysate was centrifuged at 4°C for 30 min at 48,000 × *g* to sediment most of the chromosomal DNA and cellular debris. The plasmid DNA remained in the supernatant and is referred to as a cleared lysate.

CsCl-EtBr-buoyant density gradient centrifugation. The supercoiled plasmid DNA in the cleared lysate was further purified by CsCl-ethidium bromide (EtBr)-density gradient centrifugation (26). One gram of solid CsCl and 0.1 ml of EtBr solution (5 mg of EtBr per ml in TE buffer; 10 mM Tris, 1 mM EDTA, pH 8.0) was added for each milliliter of lysate. The EtBr-DNA complex was protected from light to prevent light-activated dye nicking of the covalently closed circular (CCC) DNA. The bands were located by using long-wave ultraviolet illumination and were removed through the side of the tube with an 18-gauge needle and syringe. The dense satellite bands containing the CCC plasmid DNA were then pooled, and the EtBr was extracted three times with an equal volume of isopropanol (presaturated with water and CsCl). The samples were then dialyzed against three 1-liter changes of 15 mM NaCl and 1.5 mM sodium citrate and stored at 4°C.

Agarose gel electrophoresis. Samples of 40 to 100 μl of crude plasmid preparation were mixed with 20 μl of tracing dye solution (0.7% bromophenol blue, 7% sodium dodecyl sulfate, and 16.5% glycerol in water) and subjected to electrophoresis, similar to the method described by Meyers et al. (24). Electrophoresis was carried out in both 0.7 and 0.8% agarose (Seakem, Marine Colloids, Inc., Portland, Maine) with Tris-borate buffer (89 mM Tris base, 2.5 mM disodium EDTA, and 8.9 mM boric acid), pH 8.3, on a vertical slab gel (model 220, Bio-Rad Laboratories, Richmond, Calif.). By using a Bio-Rad model 500 power source, electrophoresis was carried out at 120 V and 20°C for 2.5 h, or until the dye reached gel bottom. For comparative purposes, samples were also electrophoresed at 30 V for 16 h. The gels were stained for 30 min in an aqueous EtBr solution (0.5 μg/ml) and then washed in water for 20 min. The DNA bands were visualized on a Transilluminator C-63 (Ultraviolet Products, San Gabriel, Calif.). The gels were photographed with a Polaroid MP-4 camera, by using Polaroid type 57 film (135-mm lens, f-16, 10 s) through combined no. 9 and no. 23A Wratten gelatin filters (Kodak).

RESULTS

Sixteen strains from the six serogroups of *L. pneumophila* were screened for the presence of plasmids. Standard methods (9, 14, 24) used for isolating plasmid DNA from *E. coli* were not effective when applied to strains of *L. pneumophila*. Extrachromosomal DNA was isolated from two strains, Atlanta-1 and -2, by using an optimized lysing and plasmid purification procedure. The elevated Triton X-100 concentration was critical for proper cell lysis and proved to be superior to Brij-58, Sarkosyl, or sodium dodecyl sulfate. Cells harvested from plates and liquid cultures gave identical results. The best resolution was obtained with 0.7% agarose gels electrophoresed at 120 V for 2.5 h at 20°C in Tris-borate buffer, pH 8.3 (Fig. 1).

Plasmids of known molecular weights, isolated from *E. coli* strain V517 were used as molecular weight markers. Plasmid pKM69 isolated from the Atlanta-1 strain and plasmid pKM70 isolated from Atlanta-2 strain both had molecular

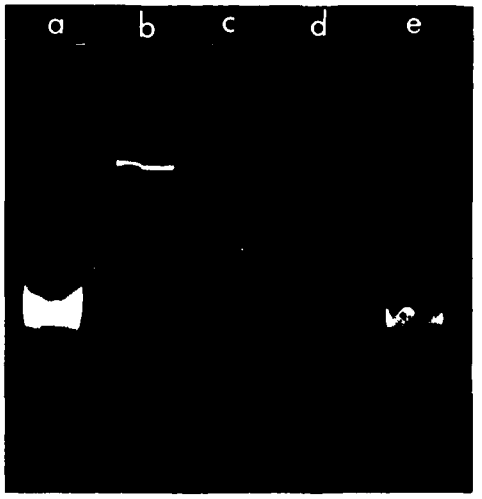

FIG. 1. *Agarose gel electrophoresis of CsCl-EtBr gradient purified plasmid DNA isolated from L. pneumophila. (a) Chromosomal DNA from Atlanta-1; (b) plasmid pKM69 from Atlanta-1; (c) plasmid pKM70 from Atlanta-2, 80 μl; (d) plasmid pKM70 from Atlanta-2, 40 μl; (e) chromosomal DNA from Atlanta-2. Electrophoresis was carried out at 120 V for 2.5 h at 20°C in 0.7% agarose, as described in the text.*

weights of ca. 30 megadaltons, based on least-squares regression plots. Spontaneous curing of these cryptic plasmids was observed after repeated subculturing on CYE agar. No significant difference in the 50% lethal dose in mice or in other phenotypic traits was detected after plasmid loss.

DISCUSSION

There are about 800,000 cases of pneumonia in the United States each year for which no known viral or bacterial agent can be identified (13). It is becoming apparent that *L. pneumophila*, and other gram-negative, weakly acid-fast bacteria (16, 17), which do not grow on commonly used media, are the etiological agents in many of these cases. Although rapid progress has been made in the diagnosis and treatment of legionellosis, there is still much to be understood concerning the genetics of *L. pneumophila*: the reason for the difference between the pneumonic and nonpneumonic forms, its precise ecological niche, its nutritional supply, and its phylogenetic relationship to other gram-negative bacteria.

This is the first report of plasmids in *Legionella*. Since we have found naturally occurring plasmids in two strains of *L. pneumophila*, it appears reasonable that this organism could support the replication of other plasmids. It is possible that the other 14 strains contain very large plasmids which are not detectable by our modified cleared lysate method (15).

In recent years, there have been epidemics of typhoid fever in Vietnam and Mexico due to *Salmonella typhi* having plasmid-mediated resistance to chloramphenicol (4), and the emergence of *Haemophilus influenzae* resistant to ampicillin and chloramphenicol (5, 20). Indigenous cryptic plasmids, such as those naturally occurring in strains of *L. pneumophila*, may acquire transposons which code for drug resistance (19). The presence of an R-plasmid coding for resistance to erythromycin would severely restrict the present treatment of legionellosis.

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